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DNA duplex with the potential to change handedness after every half a turn

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ABSTRACT

Polymorphic forms of the DNA duplex with long stretches of structural monotony are known. Several alternating purine-pyrimidine sequences have been shown to adopt left-handed Z-conformation. We report a DNA sequence d(CGCGCGATCGAT)$_n$ exhibiting alternating right-handed B and left-handed Z helical conformation after every half a turn. Further, this unusual conformation with change in handedness after every six base pairs was induced at physiological superhelical density.

INTRODUCTION

Natural DNA exhibits structural polymorphism. In addition to the various polymorphs of Watson-Crick double helices, a range of altered DNA structures encompassing left handed Z helices (1−3), cruciform (4), heteronomous DNA (5,6), bent DNA (7), parallel DNA (8) etc; have been shown to exist under defined conditions.

Several studies have shown that alternating purine-pyrimidine sequences are the most potential sequences to undergo structural transition (B to Z) with change in handedness (Review 1−3). B to Z transition has been shown to be dynamic (9) and a variety of sequences have been shown to exist in the Z conformation both in crystals (10) and under the influence of supercoiling (11,12). Recent observations of Wells et al suggest the possibility of the occurrence of such left handed helical structures *in vivo* (13). *In vitro* experiments have shown that such unusual structures could modulate key cellular processes like transcription (14,15), recombination (16) and replication (17). Formation of Z structure *in vivo* solely depends upon the possibility of stabilizing such a structure in short sequences and accommodating it within an overall B-DNA conformation. However several studies have shown that the formation of B-Z junction is energetically unfavourable and could be the key factor in inhibiting such transitions in short stretches *in vivo* (18−21). Our earlier studies with pBR322 form V DNA (22) showed that a non-B segment could alternate with a B segment within a very short stretch (fig 1). Further, in form V DNA we observed several short stretches of B and Z conformations instead of the expected long stretches of B and non-B conformations, suggesting that the junction energy need not necessarily be high for all sequences. These observations provided the impetus to investigate the possibility of such repeating units at the level of linear polymer as well as in a supercoiled circular plasmid. The possibility of an alternating right and left handed helical structure in DNA, with near zero link, was proposed from model building studies (23−25). Based on detailed theoretical studies, Sasisekharan and coworkers (26−28) showed the possibility of a right handed B helix alternating with a left handed Z helix after every half a turn with a stable link. Generation of a sequence containing short stretches of alternating B

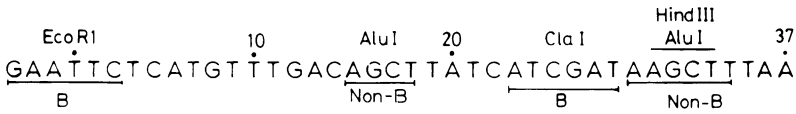

Fig 1. EcoRI to HindIII segment of pBR322 form V DNA showing altered and unaltered region as probed by restriction enzymes and methylases (8).

and non-B (like Z-form) potential sequences would provide a model system to study the conditions under which small segments of DNA would undergo structural alterations and the restrictions that would be imposed by the junctions. For such a study a polymer poly d(CGCGCGATCGAT) was synthesised which under Z favouring condition exhibited alternating right handed B and left handed Z structure after every 6bp with sharp junctions. Such an alternating right and left handed helical structure was observed even under physiological superhelical density when cloned in a supercoiled plasmid. Results presented in this communication shows the feasibility of generating a DNA duplex with near zero link where helical sense could change abruptly after every half a turn.

MATERIALS AND METHODS

All restriction enzymes, polynucleotide kinase, DNA polymerase large fragment and T4 DNA ligase were from New England Biolabs. They were used as per vendors specifications. Topoisomerase I was isolated from Wheat germ as reported earlier (29). EcoRI primer (5′ GTATCACGAGGCCCTT 3′) was synthesized on an Applied Biosystems A390 synthesizer and was a kind gift from ASTRA Research Centre, Bangalore.

Preparation of poly d(CGCGCGATCGAT): The dodecanucleotide d(CGCGCGATCGAT) was synthesised manually on solid phase using phosphoramidite approach. The oligonucleotide was purified on a 20% polyacrylamide preparative gel in the presence of 8M urea. Chemically synthesised dodecanucleotide d(CGCGCGATCGAT) was phosphorylated using γ^{32}P ATP. The kinated oligomer was annealed and the concatamers were ligated by T4 DNA ligase.

Restriction endonuclease digestion of poly d(CGCGCGATCGAT): The radiolabelled polymer was digested with HhaI, ClaI and FnuDII according to vendors specifications in the absence and presence of 100 μM hexamine cobalt chloride (HCC). The reactions were performed for one hour after which the digested mixtures were analysed on a 20% polyacrylamide gel containing 8M urea.

Construction of pRM36: Partial ClaI digest of the polymer was ligated into the ClaI site of pBR322. After transformation into *E.coli* HB101, plasmids were isolated from ampicillin resistant colonies. EcoRI, HindIII double digest of the plasmids were analysed on a 12% polyacrylamide gel and the plasmids with inserts ranging from 12 bp to 60 bp were picked up. The plasmid pRM36 used in this study contained 36bp or 3 repeats of the 12mer insert. The length and sequence of the insert was confirmed through sequencing by dideoxy method.

Two dimensional gel electrophoresis: Topoisomers of pRM36 were generated by relaxing the plasmid with topoisomerase I in the presence of different concentrations of ethidium bromide (30,31). The population of topoisomers were analysed on a 1% agarose gel (20cms×20cms) in tris borate buffer (TBE). After the run in the first dimension for 16 hrs at 2V/cm, the gel was soaked in TBE containing 750 μg/lit chloroquine for 8 hrs with gentle shaking. The run in the second dimension was for 12 hrs in TBE buffer containing 750 μg/lit chloroquine at 2V/cm.

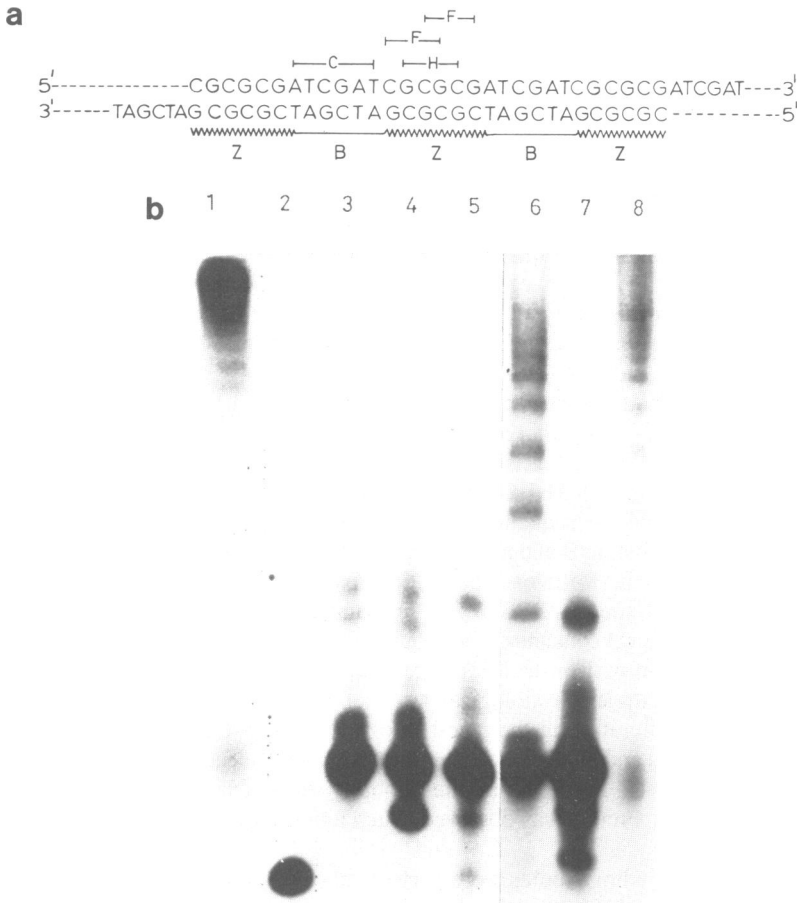

Fig 2. **(a)** Restriction enzyme sites on poly d(CGCGCGATCGAT). ClaI (C), FnuDII (F), HhaI (H) sites are shown in the repeat unit of the polymer. Right handed B-helix (– – – –) and Left handed Z-helix (vvvvvvv) potential segments.
(b) Restriction endonuclease digestion of poly d(CGCGCGATCGAT). The labelled polymer was digested with HhaI, FnuDII and ClaI and analysed on a 20% polyacrylamide gel containing 8 M urea in tris-borate buffer. Lane 1: Control polymer, 2: Octanucleotide marker, 3–5: digestion with HhaI, FnuDII and ClaI respectively, 6–8: HhaI, ClaI and FnuDII respectively in presence of 100 μM HCC.

Gel mobility retardation assay: Plasmids were incubated with affinity purified monoclonal anti Z-DNA antibody (Z-22) (a kind gift from Dr.B.D.Stollar) in a buffer containing 60mM sodium phosphate pH 8.0, 200mM sodium chloride and 30mM EDTA. After incubating at room temperature for 60 min, the samples were analysed on a 1% agarose gel in tris acetate buffer.
S1 nuclease mapping: S1 nuclease digestion of pRM36 was performed in a buffer containing 33 mM sodium acetate, 50mM sodium chloride and 0.03mM zinc chloride pH 4.5 with varying concentrations of the enzyme. To locate the site of cleavage, primer extension method of Gralla was followed (32). This method essentially involved the chain elongation

of a labelled clockwise EcoRI primer, reannealed to denatured pRM36 after nuclease digestion. The elongated primer was then analysed on a 8% sequencing gel.

RESULTS

An oligonucleotide d(CGCGCGATCGAT) containing B and Z helicogenic segments was synthesised. CD studies showed the presence of partial Z conformation in the concatamer of the dodecamer unit under Z-favouring conditions (33). Since the polymer of the dodecamer had intrinsically many restriction sites (fig 2a), we made use of the structure sensitivity of the restriction enzymes (34−36) to probe every base of the dodecamer unit. ClaI, FnuDII and HhaI, whose recognition sequences fall within the B or Z helicogenic region, were used in this study. Fig 2b shows the restriction enzyme digestion pattern of the ^{32}P labelled polymer in the presence and absence of 100 μM HCC which is one of the most potential inducers of Z-conformation. In the absence of HCC the polymer was digested by all the three enzymes and gave the expected fragments. In the presence of 100 μM HCC, FnuDII and HhaI were unable to digest the polymer whereas ClaI cleaved it efficiently. However ClaI, FnuDII and HhaI were found to digest pBR322 efficiently even in the presence of 500μM HCC (data not shown). FnuDII and HhaI do not cleave their respective recognition sequences when in the Z-form (34,35).

To see whether alternating B and non-B conformation (Z-form) could be generated within small segments under the force of supercoiling, partial Cla I digest of the polymer was inserted into the Cla I site of pBR322 (fig 3). Analysis of the plasmid pRM36 (with 36 bp insert) in an agarose gel containing chloroquine showed a shift in the gaussian distribution of the plasmids in comparison to the parent plasmid. This is indicative of a structural transition in the insert under physiological superhelical density. To determine the type and extent of structural alteration in the insert, a distribution of topoisomers of the plasmid pRM36 was generated and analysed by two dimensional gel electrophoresis in the presence of chloroquine (fig 4). A break in the mobility of the topoisomers (at γ −0.04) corresponding to 3 supercoils was observed. Such a break was not observed in the parent plasmid.

Z-DNA antibody binding studies were performed (fig 5) to confirm the presence of Z-conformation in the pRM36 plasmid. In the presence of monoclonal Z-DNA antibody (Z-22), the electrophoretic mobility of the supercoiled form of the plasmid pRM36 was retarded, whereas the parent plasmid pBR322 showed no change in mobility (fig 5). The

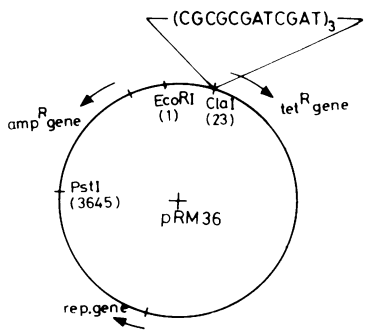

Fig 3 : Plasmid pRM36—a derivative of pBR322 containing the 36bp insert in the ClaI site.

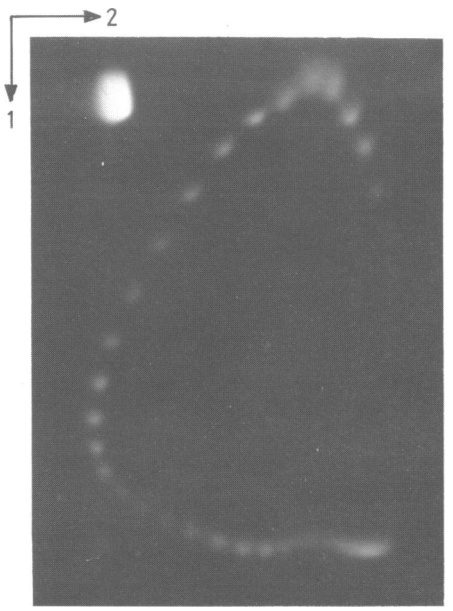

Fig 4: Two dimensional gel electrophoresis of pRM36.

dimer supercoiled pRM36 plasmid which has a similar mobility to that of the monomer open circular species, was also found to be retarded in the presence of Z-22 antibody. The supercoiled nature of the retarded dimer was confirmed by 2D gel electrophoresis (37). The electroeluted monomer supercoiled plasmid showed retardation without any shift in the mobility of the open circular species (data not shown). pLP332 containing a $(CG)_{16}$ insert, which is known to exist in the Z-conformation was also retarded.

B -- Z junctions have been shown to be sensitive to S1 nuclease (38). S1 nuclease mapping

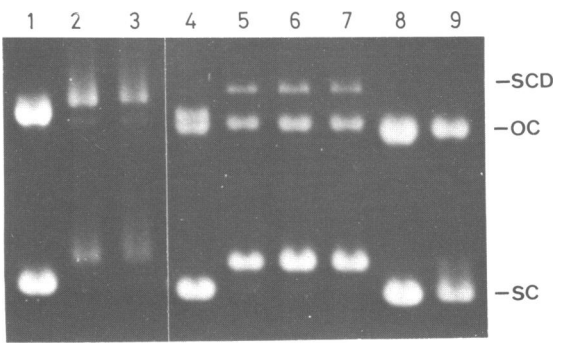

Fig 5: Gel mobility retardation assay of plasmids pRM36, pBR322, pLP332 in the presence of affinity purified anti Z-DNA antibody (Z-22). Lanes 1–3: pLP332 with 0, 0.35 and 0.7 μg of antibody, 4–7: pRM36 with 0, 0.35, 0.70, and 1.05 μg of antibody, 8 and 9: pBR322 with 0 and 0.7 μg of antibody. OC = Open Circle; SC = Supercoiled; SCD = Retarded Supercoiled Dimer.

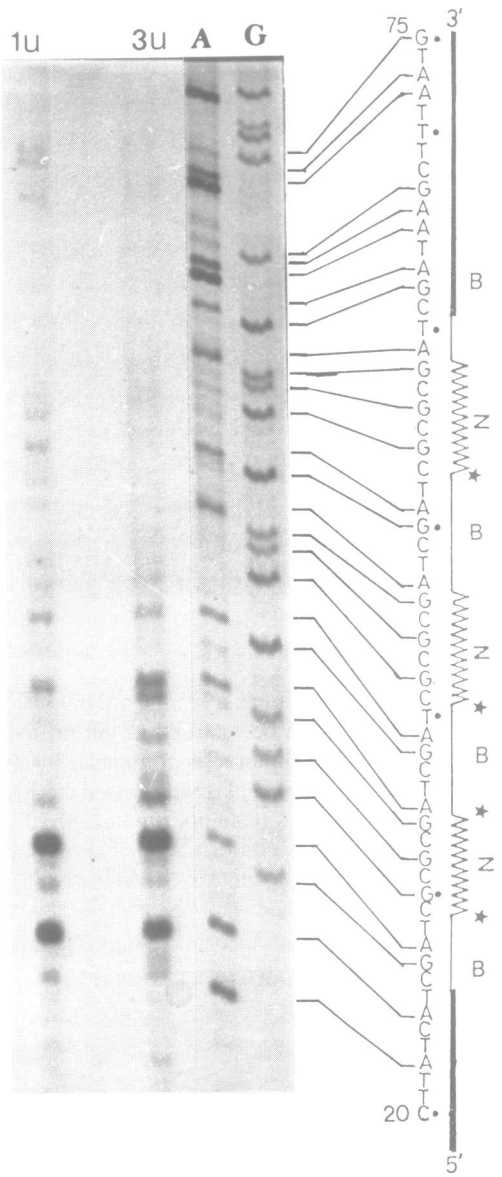

Fig 6: Fine mapping of S1 nuclease hypersensitive sites in pRM36. (*) represents the junction region of B and Z form which showed hypersensitivity to S1 nuclease. S1 nuclease digested materials were run in parallel with a dideoxy sequence ladder (A and G).

of the supercoiled pRM36 plasmid was performed to locate the junctions of B and Z conformation. Fig 6 shows the autoradiogram of the S1 nuclease fine mapping of the insert region in supercoiled pRM36 under different enzyme concentrations. Fine mapping gel

showed strong cleavage near the junctions between the (CGCGCG) and (ATCGAT) segments. Of the six possible B-Z junctions, 4 of them, at positions 29,35, 41, and 53 showed hypersensitivity.

DISCUSSION

B to Z transition in alternating purine-pyrimidine sequences have been shown to be an all or none process (20,39,40). In an earlier study with a highly supercoiled form V DNA, we observed the presence of an unusual structure containing alternating B and non-B conformation (probed by restriction endonucleases and methylases) alternating 4 times within a region of 40 bp starting from the EcoRI site in pBR322. One of the segments (ATCGAT) remained in the B-conformation even when flanked by sequences in the non-B conformation. Extensive studies on synthetic oligo and polynucleotides have shown that (CGCGCG) adopts Z-conformation under a variety of conditions (10,41,42). Considering the above observations an oligonucleotide d(CGCGCGATCGAT) was designed to generate a polymer with alternating Z and B favouring segments after every half a turn, to have both the conformations with a stable link in a single polynucleotide.

CD measurements of the concatamer solution under Z favouring condition (5M NaCl), showed the presence of 50% Z-conformation in the dodecamer unit (33). Since spectroscopic methods cannot distinguish whether 50% of the population or 50% of the sequence is in the Z conformation, enzymatic probes were used to locate regions with B and Z conformation in the polymer. FnuDII and HhaI do not cleave the recognition site if they exist in the Z-form. Since HCC is known to induce Z-conformation in (CGCGCG) (40,43), resistance of poly d(CGCGCGATCGAT) to digestion by FnuDII and HhaI, in the presence of 100 μM HCC, is indicative of structural alteration within the (CGCGCG) segment. The (ATCGAT) segment remained sensitive to ClaI cleavage even in the presence of 100 μM HCC showing the presence of B-conformation. Since (CGCGCG) sequence forms 50% of the polymer and further is resistant to cleavage by FnuDII and HhaI in the presence of HCC, the polymer could be visualized to adopt a conformation which alternates between left handed Z conformation and right handed B conformation under Z-favouring condition after every six base pairs. Various studies with enzymatic probes have indicated that the B-Z junction extends to 5bp (44,45). Our studies with pBR322 form V DNA (20) have shown that B-Z junctions can be very sharp (much less than 5bp). The accessibility of ClaI site abutting the Z segment (FnuDII and HhaI sites) in condition favouring left handed Z-conformation indicates that the B-Z junction is sharp in this case.

To find out whether such alternating B-Z structure could be stabilized under physiological conditions, a plasmid pRM36 containing $(CGCGCGATCGAT)_3$ insert was used for further studies. Structural transition induced under torsional stress would lead to a change in the twist of the molecule, the extent of the reduction, which is indicative of the kind and extent of structural alteration can be monitored. The change in supercoiling suggests a structural alteration involving a change in the twist which affects the overall writhe of the plasmid. A change of 3 superhelical turns, resulting from structural transition within the insert of supercoiled pRM36, as observed in two dimensional gel analysis, could be interpreted either as a cruciform extrusion of 36bp or a left handed Z-conformation of about 18bp in the insert. Cruciform extrusion in the insert is unlikely due to the kinetic barrier brought about by the high GC content (46). To confirm the presence of supercoil induced Z-conformation in the insert, Z-DNA antibody binding studies were carried out. Significant binding of Z-22 antibody was observed with pRM36 as well as pLP332. The

absence of any binding to pBR322, the parent plasmid, provides strong evidence for the presence of Z-structure within the insert. This supports the conclusions drawn from the two dimensional gel analysis wherein 18bp of the insert was thought to undergo B to Z transition. Hypersensitivity observed from S1 mapping indicates the presence of multiple B--Z junctions in the insert region since B-Z junctions are known to be S1 hypersensitive. These hypersensitive sites occur nearly after every 6bp from the 5′ to 3′ direction of the insert in pRM36.

Z-DNA antibody binding, analysis of topoisomers by two dimensional gel, resistance of the (CGCGCG) segments to FnuDII and HhaI digestion under Z-favouring condition and S1 nuclease hypersensitivity to junction regions, shows that $(CGCGCGATCGAT)_n$ sequence exists in alternating B and Z conformation with sharp junctions. Such a novel and unusual structure could also be stabilized under physiological superhelical density in a circular plasmid. Thus it could be shown that right and left handed helical segments could exist in alternation after every half a turn of the helix. The resultant conformation of such a DNA sequence will closely resemble the RL model of DNA, with near zero link, as proposed earlier (28)

The unusual structure involving change in handedness within short stretches could occur in natural DNA in small segments under physiological superhelical density provided the base sequence requirements are met. Z-conformation has been shown to favour the recombination event (16). It is tempting to speculate that DNA segments having such alternating B-Z structure, with near zero link, may act as 'hot spots' for recombination. Will such topologically unlinked regions favour open complex formation with RNA polymerase? It would be interesting to test the effect of such unusual structural cassettes on transcriptional initiation.

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